(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 August 2003 (21.08.2003)

PCT

(10) International Publication Number
WO 03/068976 A2

(51) International Patent Classification[7]: C12N 15/56, C12P 19/14, 19/18, 19/20, 19/22, 7/06

(21) International Application Number: PCT/DK03/00084

(22) International Filing Date: 10 February 2003 (10.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2002 00227 14 February 2002 (14.02.2002) DK
PA 2002 01291 2 September 2002 (02.09.2002) DK

(71) Applicant *(for all designated States except US)*: **NOVOZYMES A/S** [DK/DK]; Krogshøjvej 36, DK-2880 Bagsværd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NORMAN, Barrie, Edmund** [GB/DK]; Magnoliavej 5B, DK-3460 Birkerød (DK). **VIKSØ-NIELSEN, Anders** [DK/DK]; Lindevej 12, Jørlunde, DK-3550 Slangerup (DK). **OLSEN, Hans, Sejr** [DK/DK]; Højbjerg Vang 7, DK-2840 Holte (DK). **PEDERSEN, Sven** [DK/DK]; Emil Reesens Vej 9, DK-2820 Gentofte (DK).

(74) Common Representative: **NOVOZYMES A/S**; Patents, Krogshøjvej 36, DK-2880 Bagsværd (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/068976 A2

(54) Title: STARCH PROCESS

(57) Abstract: The present invention relates to a process for enzymatic hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

STARCH PROCESS

FIELD OF THE INVENTION

The present invention relates to a one step process for hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

BACKGROUND OF THE INVENTION

A large number of processes have been described for converting starch to starch hydrolysates, such as maltose, glucose or specialty syrups, either for use as sweeteners or as precursors for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products.

Starch is a high molecular-weight polymer consisting of chains of glucose units. It usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains of alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. In the case of converting starch into a soluble starch hydrolysate, the starch is depolymerized. The conventional depolymerization process consists of a gelatinization step and two consecutive process steps, namely a liquefaction process and a saccharification process.

Granular starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation. During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase. The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The temperature is then lowered to 60°C, a glucoamylase or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase are added, and the saccharification process proceeds for about 24 to 72 hours.

It will be apparent from the above discussion that the conventional starch conversion process is very energy consuming due to the different requirements in terms of temperature during the various steps. It is thus desirable to be able to select the enzymes used in the process so that the overall process can be performed without having to gelatinize the starch.

Such processes are the subject for the patents US4591560, US4727026 and US4009074 and EP0171218.

The present invention relates to a one-step process for converting granular starch into soluble starch hydrolysate at a temperature below initial gelatinization temperature of the starch.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a one step process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the simultaneous action of the following enzyme activities, a first enzyme which is a member of the Glycoside Hydrolase Family 13, has alpha-1.4-glucosidic hydrolysis activity and comprises a Carbohydrate-Binding Module of Family 20, and a second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), or an glucoamylase (E.C.3.2.1.3).

In a second aspect the invention provides a process for production of high fructose starch-based syrup (HFSS), the process comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for conversion of the soluble starch hydrolysate into a of high fructose starch-based syrup (HFSS).

In a third aspect the invention provides a process for production of fuel or potable ethanol; comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for fermentation of the soluble starch hydrolysate into ethanol, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "granular starch" is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization. Starch is formed in plants as tiny granules insoluble in water. These granules are preserved in starches at temperatures below the initial gelatinization temperature. When put in cold water, the grains may absorb a small amount of the liquid. Up to 50°C to 70°C the swelling is reversible, the degree of reversibility being dependent upon the particular starch. With higher temperatures an irreversible swelling called gelatinization begins.

The term "initial gelatinization temperature" is understood as the lowest temperature at which gelatinization of the starch commences. Starch begins to gelatinize between 60°C and 70°C, the exact temperature dependent on the specific starch. The initial gelatinization

temperature depends on the source of the starch to be processed. The initial gelatinization temperature for wheat starch is approximately 52°C, for potato starch approximately 56°C, and for corn starch approximately 62°C. However, the quality of the starch initial may vary according to the particular variety of the plant species as well as with the growth conditions and therefore initial gelatinization temperature should be determined for each individual starch lot.

The term "soluble starch hydrolysate" is understood as the soluble products of the processes of the invention and may comprise mono- di-, and oligosaccharides, such as glucose, maltose, maltodextrins, cyclodextrins and any mixture of these. Preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

The term "Speciality Syrups", is an in the art recognised term and is characterised according to DE and carbohydrate spectrum (See the article "New Speciality Glucose Syrups", p. 50+, in the textbook "Molecular Structure and Function of Food Carbohydrate", Edited by G.G. Birch and L.F. Green, Applied Science Publishers LTD., London). Typically Speciality Syrups have a DE in the range from 35 to 45.

The "Glycoside Hydrolase Family 13" is in the context of this invention defined as the group of hydrolases comprising a catalytic domain having a (beta/alpha)8 or TIM barrel structure and acting on starch and related substrates through an alpha-retaining reacting mechanism (Koshland, 1953, Biol.Rev.Camp.Philos.Soc 28, 416-436).

The enzymes having "alpha-1.4-glucosidic hydrolysis activity" is in the context of this invention defined as comprising the group of enzymes which catalyze the hydrolysis and/or synthesis of alpha-1,4-glucosidic bonds as defined by Takata (Takata et al, 1992, J. Biol. Chem. 267, 18447-18452) and by Koshland (Koshland, 1953, Biol.Rev. Camp. Philos. Soc 28, 416-436).

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683.

Enzymes which; (a) are members of the Glycoside Hydrolase Family 13; (b) have alpha-1.4-glucosidic hydrolysis activity and (c) comprise a Carbohydrate-Binding Module of Family 20, and are specifically contemplated for this invention comprise the enzymes classified as EC 2.4.1.19, the cyclodextrin glucanotransferases, and EC 3.2.1.133, the maltogenic alpha-amylases, and selected members of 3.2.1.1 the alpha-amylases, and 3.2.1.60, the maltotetraose-forming amylases.

The "hydrolysis activity" of CGTases and maltogenic alpha-amylases is determined by measuring the increase in reducing power during incubation with starch according to Wind, R.D. et al 1995 in Appl. Environ. Microbiol.61:1257-1265. Reducing sugar concentrations is measured with the dinitrosalisylic acid method according to Bernfield (Bernfield, P. 1955. Amylases alpha and beta. Methods Enzymol. 1:149-158), with a few modifications. Diluted enzyme is incubated for an appropriate period of time with 1% (wt/v) soluble starch (Paselli SA2 starch from Avebe, The Netherlands or alternatively soluble starch from Merck) in a 10 mM sodium citrate (pH 5.9) buffer at 60°C. One unit of hydrolysis activity is defined as the amount of enzyme producing 1 micro mol of maltose per minute under standard conditions.

The polypeptide "homology" referred to in this disclosure is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Cyclodextrin glucanotransferases (CGTases)

A particular enzyme to be used as a first enzyme in the processes of the invention may be a cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19). Cyclomaltodextrin glucanotransferase, also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, in the following termed CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins of various sizes. Most CGTases have both transglycosylation activity and starch-degrading activity. Contemplated CGTases are preferably of microbial origin, and most preferably of bacterial origin. Specifically contemplated CGTases include the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the sequence shown as amino acids 1 to 679 of SEQ ID NO:2 in WO02/06508, the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence of the polypeptide disclosed in Joergensen et al, 1997 in figure 1 in Biotechnol. Lett. 19:1027-1031, and the CGTases described in US5278059 and US5545587. Preferably the CGTase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9,10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per min/mg. CGTases may be added in amounts of 0.01-100.0 NU/g DS, preferably from 0.2-50.0 NU/g DS, preferably 10.0-20.0 NU/g DS.

Maltogenic alpha-amylase

Another particular enzyme to be used as a first enzyme in the processes of the invention is a maltogenic alpha-amylase (E.C. 3.2.1.133). Maltogenic alpha-amylases (glucan 1,4-alpha-maltohydrolase) are able to hydrolyse amylose and amylopectin to maltose in the alpha-configuration. Furthermore, a maltogenic alpha-amylase is able to hydrolyse maltotriose as well as cyclodextrins. Specifically contemplated maltogenic alpha-amylases may be derived from *Bacillus* sp., preferably from *Bacillus stearothermophilus*, most preferably from *Bacillus stearothermophilus* C599 such as the one described in EP120.693. This particular maltogenic alpha-amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628. A preferred maltogenic alpha-amylase has an amino acid sequence having at least 70% identity to amino acids 1-686 of SEQ ID NO:1 in US6162628, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99%. Most preferred variants of the maltogenic alpha-amylase comprise the variants disclosed in WO99/43794.

The maltogenic alpha-amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628 has a hydrolysis activity of 714. Preferably the maltogenic alpha-amylase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 5, 6, 7, 8, 9,10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.

Maltogenic alpha-amylases may be added in amounts of 0.01-40.0 MANU/g DS, preferably from 0.02-10 MANU/g DS, preferably 0.05-5.0 MANU/g DS.

Fungal alpha-amylase

A particular enzyme to be used as a second enzyme in the processes of the invention is a fungal alpha-amylase (EC 3.2.1.1), such as a fungamyl-like alpha-amylase. In the present disclosure, the term "fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high homology, i.e. more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID No. 10 in WO96/23874. Fungal alpha-amylases may be added in an amount of 0.001-1.0 AFAU/g DS, preferably from 0.002-0.5 AFAU/g DS, preferably 0.02-0.1 AFAU/g DS.

Beta-amylase

Another particular enzyme to be used as a second enzyme in the processes of the invention may be a beta-amylase (E.C 3.2.1.2). Beta-amylase is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers.

Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These

beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7.0. Contemplated beta-amylase include the beta-amylase from barley Spezyme® BBA 1500, Spezyme® DBA and Optimalt™ ME, Optimalt™ BBA from Genencor int as well as Novozym™ WBA from Novozymes A/S.

Glucoamylase

A further particular enzyme to be used as a second enzyme in the processes of the invention may also be a glucoamylase (E.C.3.2.1.3) derived from a microorganism or a plant. Preferred is glucoamylases of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO92/00381 and WO00/04136; the *A. awamori* glucoamylase (WO84/02921), A. oryzae (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), *Prot. Engng.* 9, 499-505); D257E and D293E/Q (Chen et al. (1995), *Prot. Engng.* 8, 575-582); N182 (Chen et al. (1994), *Biochem. J.* 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein *Engng.* 10, 1199-1204. Furthermore Clark Ford presented a paper on Oct 17, 1997, ENZYME ENGINEERING 14, Beijing/China Oct 12-17, 97, Abstract book p. 0-61. The abstract suggests mutations in positions G137A, N20C/A27C, and S30P in an *Aspergillus awamori* glucoamylase to improve the thermal stability. Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO99/28448), *Talaromyces leycettanus* (US patent no. Re.32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215). Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP135,138), and *C. thermohydrosulfuricum* (WO86/01831). Preferred glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136. Also contemplated are the commercial products AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (from Novozymes); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900 (from Enzyme Bio-Systems); G-ZYME™ G990 ZR (*A. niger* glucoamylase and low protease content).

Glucoamylases may be added in an amount of 0.02-2.0 AGU/g DS, preferably 0.1-1.0 AGU/g DS, such as 0.2 AGU/g DS.

Additional enzymes.

The processes of the invention may also be carried out in the presence of a third enzyme. A particular third enzyme may be a *Bacillus* alpha-amylase (often referred to as

"Termamyl-like alpha-amylases"). Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available as Termamyl), *B. amyloliquefaciens,* and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO96/23874, WO97/41213, and WO99/19467. Specifically contemplated is a recombinant *B.stearothermophilus* alpha-amylase variant with the mutations: I181* + G182* + N193F. *Bacillus* alpha-amylases may be added in effective amounts well known to the person skilled in the art.

Another particular third enzyme of the process may be a debranching enzyme, such as an isoamylase (E.C. 3.2.1.68) or a pullulanases (E.C. 3.2.1.41). Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by the limited action on alpha-limit dextrins. Debranching enzyme may be added in effective amounts well known to the person skilled in the art.

Embodiments of the invention

The starch slurry to be subjected to the processes of the invention may have 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids granular starch.

After being subjected to the process of the first aspect of the invention at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or preferably 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

According to the invention the processes of the first and second aspect is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which the processes are conducted is at least 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, or preferably at least 60°C.

The pH at which the process of the first aspect of the invention is conducted may in be in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

The exact composition of the products of the process of the first aspect of the invention, the soluble starch hydrolysate, depends on the combination of enzymes applied as well as the type of granular starch processed. Preferably the soluble hydrolysate is maltose with a purity of at least 85%, 90%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Even more preferably the soluble starch hydrolysate is glucose, and most

preferably the starch hydrolysate has a DX (glucose percent of total solubilised dry solids) of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Equally contemplated, however, is the process wherein the product of the process of the invention, the soluble starch hydrolysate, is a speciality syrup, such as a speciality syrup containing a mixture of glucose, maltose, DP3 and DPn for use in the manufacture of ice creams, cakes, candies, canned fruit.

The granular starch to be processed in the processes of the invention may in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically the granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley. The granular starch to be processed may be a highly refined starch quality, preferably more than 90%, 95%, 97% or 99.5 % pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibres. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where the starch hydrolysate is used in production of syrups. Both dry and wet milling is well known in the art of starch processing and are equally contemplated for the processes of the invention.The process of the first aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Also contemplated is the process conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

In the process of the second aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS). This conversion is preferably achieved using a glucose isomerase, and more preferably by an immobilized glucose isomerase supported on a solid support. Contemplated isomerases comprises the commercial products Sweetzyme™ IT from Novozymes A/S, G -zyme™ IMGI and G-zyme™ G993, Ketomax™ and G-zyme™ G993 from Rhodia, G-zyme™ G993 liquid and GenSweet™ IGI from Genemcor Int.

In the process of the third aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is used for production of fuel or potable ethanol. In the process of the third aspect the fermentation may be carried out simultaneously or separately/sequential to the hydrolysis of the granular starch slurry. When the fermentation is performed simultaneous to the hydrolysis the temperature is preferably between 30°C and 35°C, and more preferably between 31°C and 34°C. The process of the third aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

MATERIALS AND METHODS

Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue colour is formed, but during the break-down of the starch the blue colour gets weaker and gradually turns into a reddish-brown, which is compared to a coloured glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5.26 g starch dry substance Merck Amylum solubile.

A folder AF 9/6 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

CGTase activity (KNU)

The CGTase alpha-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl2, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50

mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temperature, pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

A folder EAL-SM-0351 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference

Maltogenic alpha-amylase activity (MANU)

One Maltogenic Amylase Novo Unit (MANU) is defined as the amount of enzyme which under standard will cleave one micro mol maltotriose per minute. The standard conditions are 10 mg/ml maltotriose, 37°C, pH 5.0, and 30 minutes reaction time. The formed glucose is converted by glucose dehydrogenase (GlucDH, Merck) to gluconolactone under formation of NADH, which is determined spectophotometrically at 340 nm. A folder (EAL-SM-0203.01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A folder (AEL-SM-0131) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Fungal alpha-amylase activity (FAU)

The alpha-amylase activity is measured in FAU (Fungal Alpha-Amylase Units). One (1) FAU is the amount of enzyme which under standard conditions (i.e. at 37°C and pH 4.7) breaks down 5260 mg solid starch (Amylum solubile, Merck) per hour. A folder AF 9.1/3, describing this FAU assay in more details, is available upon request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (from Novozymes A/S, glucoamylase wildtype Aspergillus niger G1, also disclosed in Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102 and in WO92/00381). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with the following description. In this method 1 AFAU is defined as the amount of enzyme, which degrades 5.26 mg starch dry solids per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

	Alpha-amylase	
Starch + Iodine	→	Dextrins + Oligosaccharides
	40°C, pH 2.5	
Blue/violet	t=23 sec.	Decoloration

Standard conditions/reaction conditions: (per minute)

Substrate:	starch, approx. 0.17 g/L
Buffer:	Citate, approx. 0.03 M
Iodine (I2):	0.03 g/L
CaCl2:	1.85 mM
pH:	2.50 - 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	lambda=590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

If further details are preferred these can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, and incorporated by reference.

Beta-amylase activity (DP°)

The activity of SPEZYME® BBA 1500 is expressed in Degree of Diastatic Power (DP°). It is the amount of enzyme contained in 0.1 ml of a 5% solution of the sample enzyme preparation that will produce sufficient reducing sugars to reduce 5 ml of Fehling's solution when the sample is incubated with 100 ml of substrate for 1 hour at 20°C.

Pullulanase activity (New Pullulanase Unit Novo (NPUN)

Pullulanase activity may be determined relative to a pullulan substrate. Pullulan is a linear D-glucose polymer consisting essentially of maltotriosyl units joined by 1,6-alpha-links. Endo-pullulanases hydrolyze the 1,6-alpha-links at random, releasing maltotriose, 6^3-alpha-maltotriosyl-maltotriose, 6^3-alpha-(6^3-alpha-maltotriosyl-maltotriosyl)-maltotriose.

One new Pullulanase Unit Novo (NPUN) is a unit of endo-pullulanase activity and is measured relative to a Novozymes A/S Promozyme D standard. Standard conditions are 30 minutes reaction time at 40°C and pH 4.5; and with 0.7% pullulan as substrate. The amount of red substrate degradation product is measured spectrophotometrically at 510 nm and is proportional to the endo-pullulanase activity in the sample. A folder (EB-SM.0420.02/01) describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Under the standard conditions one NPUN is approximately equal to the amount of enzyme which liberates reducing carbohydrate with a reducing power equivalent to 2.86 micromole glucose per minute.

Determination of CGTase hydrolysis activity

The CGTase hydrolysis activity was determined by measuring the increase in reducing power during incubation with Paselli SA2 starch (from Avebe, The Netherlands) as described by Wind et al. 1995 in Appl. Environ. Microbiol. 61: 1257-1265.

Determination of sugar profile and solubilised dry solids

The sugar composition of the starch hydrolysates was determined by HPLC and glucose yield was subsequently calculated as DX. °BRIX, solubilised (soluble) dry solids of the starch hydrolysates were determined by refractive index measurement.

Materials

The following enzyme activities were used. A maltogenic alpha-amylase with the amino acid sequence shown in SEQ ID No: 1 in WO9/943794. A glucoamylase derived from *Aspergillus oryzae* having the amino acid sequence shown in WO00/04136 as SEQ ID No: 2 or one of the disclosed variants. An acid fungal alpha-amylase derived from *Aspergillus niger*. A *Bacillus* alpha-amylase which is a recombinant *B.stearothermophilus* variant with the mutations: I181*+ G182*+N193F. A fungal alpha-amylase derived from *Aspergillus oryzae*. A CGTase N with the sequence shown herein as SEQ ID NO 1. A CGTase O with the sequence shown herein as SEQ ID NO 2. A CGTase T with the amino acid sequence disclosed in figure 1 in Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031 and shown herein as SEQ ID NO 3. A CGTase A having the sequence shown herein as SEQ ID NO 4.

Common corn starch (C x PHARM 03406) was obtained from Cerestar.

Example 1

This example illustrates the conversion of granular starch into glucose using CGTase T and a glucoamylase and an acid fungal amylase. A slurry with 33% dry solids (DS) granular starch was prepared by adding 247.5 g of common corn starch under stirring to 502.5 ml of water. The pH was adjusted with HCl to 4.5. The granular starch slurry was distributed to 100 ml blue cap flasks with 75 g in each flask. The flasks were incubated with magnetic stirring in a 60°C water bath. At zero hours the enzyme activities given in table 1 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours.

Table 1. The enzyme activity levels used were:

CGTase T KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
12.5	200	50
25.0	200	50
100.0	200	50

Total dry solids starch was determined using the following method. The starch was completely hydrolyzed by adding an excess amount of alpha-amylase (300 KNU/Kg dry solids) and subsequently placing the sample in an oil bath at 95 °C for 45 minutes. After filtration through a 0.22 microM filter the dry solids was measured by refractive index measurement.

Soluble dry solids in the starch hydrolysate were determined on samples after filtering through a 0.22 microM filter. Soluble dry solids were determined by refractive index measurement and the sugar profile was determined by HPLC. The amount of glucose was calculated as DX. The results are shown in table 2 and 3.

Table 2. Soluble dry solids as percentage of total dry substance at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	68	82	89	94
25.0	76	89	93	97
100.0	83	96	98	99

Table 3. The DX of the soluble hydrolysate at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	92.6	94.5	95.1	95.3
25.0	92.4	94.8	95.4	95.5
100.0	92.7	94.9	95.4	95.4

Example 2

This example illustrates the conversion of granular starch into glucose using CGTase T, a glucoamylase, an acid fungal alpha-amylase and a Bacillus alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzymes activities given in table 4 were dosed to the flask.

Table 4. The enzyme activity levels used were:

CGTase T KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS	*Bacillus* alpha-amylase KNU/kg DS
5.0	200	50	300

Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 4 and 5.

Table 5. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
82.8	93.0	96.3	98.7

Table 6. The DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
92.8	94.9	95.5	95.8

Example 3

This example illustrates the conversion of granular starch into glucose using a maltogenic alpha-amylase, a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 6 were dosed to the flasks.

Table 6. The enzyme activity levels used were:

	Maltogenic alpha-amylase MANU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
Flask 1	5000	200	50
Flask 2	20000	200	50

Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 7 and 8.

Table 7. Soluble dry solids as percentage of total dry substance at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	63.1	75	79.3	85.3
20000	67.0	77.9	82.7	88.1

Table 8. The DX of the soluble hydrolysate at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	95.2	95.4	95.3	95.5
20000	93.8	94.9	94.9	94.8

Example 4

This example illustrates the only partial conversion of granular starch into glucose using a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 9 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours. The samples were analyzed as described in examples 1. The results are shown in table 10 and 11.

Table 9. The enzyme activity level used were:

Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
200	50

Table 10. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
28.5	36.3	41.6	45.7

Table 11. DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
27.7	34.9	39.2	42.2

Example 5

This example illustrates the correlation between the hydrolysis activity of four different CGTases (CGTase A, CGTase N, CGTase O and CGTase T) versus the yield during conversion of granular starch into glucose syrup using a CGTase and a glucoamylase measured as soluble dry solids and development in DX.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hour the CGTases were all dosed at 100 KNU/kg DS in combination with glucoamylase at 200 AGU/kg DS. Samples were withdrawn at 48 hours and analyzed as described in examples 1. Results are presented in table 12.

Table 12. Hydrolysis activity (micro mol per min/mg protein), and soluble dry solids (DS) and DX after 48 hors

CGTase	Hydrolysis act.	Soluble DS	DX
CGTase N	0.27	37.4	35.1
CGTase A	0.38	49.9	46.7
CGTase O	1.62	60.9	57.1
CGTase T	4.59	97.9	91.2

Example 6

This example illustrates the process conducted in an ultrafiltration system where the retentate was held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. A slurry comprising 100 kg granular corn starch suspended in 233 L tap city water and CGTase T (12.5 KNU/kg starch), *Bacillus* alpha-amylase (300 KNU/kg starch) and glucoamylase (200 AGU/kg starch) was processed in a batch ultrafiltration system (type PCI) with a tubular membrane module (type PU 120). The slurry was stirred at 100 rpm, pH was adjusted to 4.5 using 170 mL of 30 % HCl, and the reaction temperature was set at 57°C.

Samples of permeate and retentate were analyzed for dry solids content and for sugar composition.

The correction factor for non soluble material is: q = (100-S%)/(100-°BRIX). The centrifugation index for sugar is: ciS% = °BRIX/S% (no correction). The theoretical yield of sugar (glucose) S_{yield} = ciS%*q*100/111*100 %. A correction has thus been done for 100 kg starch dry matter giving ca. 111 kg glucose dry matter as a result of the hydrolysis reaction.

A trial was made in a simple batch system using the same enzyme system as for the membrane trial. As the comparison in table 15 a and b shows the membrane system reached the maximal solubilisation of starch earlier.

Table 13. Dry solids content and sugar composition of retentate and permeate

Sample	Hours	Reactor volume, L	% DS	% DP1	% DP2	% DP3	% DP4
Reactor	3	207	16.1	75.3	10.3	2.6	11.5
Reactor	28	123	28.3	95.0	2.7	0.8	1.5
Reactor	53	123	31.4	95.2	3.4	0.5	0.9
Permeate	3	207	12.1	71.2	17.4	2.9	8.5
Permeate	28	123	21.8	94.9	2.9	0.8	1.3

Tabel 14. Dry solids distribution in retentate at 3, 28, 53 and 77 hours.

	3 hours	28 hours	53 hours	77 hours
Soluble DS	16	28	31	39
Total DS	38	37	42	45

Table 15 a. Theoretical yield of glucose versus time for the membrane system

Hours	% total DS in the reactor	°BRIX	q=(100-S%)/(100-°Brix)	cis%=°Brix/S%	Theoretical yield sis=cis*q*100/111%
0	27.0	2.2	0.75	0.08	5
24	35.9	27.3	0.88	0.76	73
48	41.2	30.0	0.84	0.73	89
72	41.2	33.1	0.88	0.80	98
94	41.2	34.8	0.90	0.85	103

Table 15 b. Theoretical yield of glucose versus time for a batch reactor system.

Hours	% total DS in the reactor	°BRIX	q=(100-S%)/(100-°Brix)	cis%=°Brix/S%	Theoretical yield sis=cis*q*100/111%
0	29.7	2.0	0.72	0.07	4.
24	29.7	25.6	0.95	0.86	74
48	29.7	28.8	0.99	0.97	86
72	29.7	29.8	1.00	1.00	91
94	29.7	29.8	1.00	1.00	91

The conclusion was that when substrate saturation was maintained during the saccharification in a membrane system the degree of solubilization was improved compared to a simple batch reactor system for cold saccharification of raw starch.

Example 7

This example illustrates a simultaneous cold liquefaction and saccharification process of the invention carried out in a continuous working microfiltration membrane reactor using a ceramic module.

A 200 L feed mixer tank was connected by a reactor feed pump to a 200 L reactor tank with temperature control. Using a pump with a capacity of 0-20 l/h the mixture from the reactor was recycled through a APV ceramic microfiltration module for separation of glucose. Pore size was 0.2 micro m and the membrane area was 0.2 m2.

The reactor worked for about 200 hours using a dosage of 100 KNU/kg DS CGT-ase T and 300 AGU/kg DS of glucoamylase. With an average holding time in the reactor of 35-45 hours the system operated at steady state for the full period producing a DP1= 93 % glucose syrup at a yield of close to 100 %.

The reactor tank was loaded with 60 kg of corn starch type Cerestar C x PHARM 03406 suspended in 140 L of tap city water of 58°C under stirring. Using the steam heated mantel the temperature was adjusted to 60°C. Using 30 % HCl the pH was lowered from 6.1 to 4.5. The pH was re-checked (pH=4.5) aft er 15 minutes. At zero hours, immediately before adding the enzymes, CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch), samples were taken for determination of % sludge volume after centrifugation at 3000 rpm for 3 min in a table centrifuge. Furthermore the °BRIX of the supernatant was measured using a refractometer. The course of the reaction was followed regularly by measurements of sludge volumes and °BRIX of the supernatants as described above.

The feed mixer tank was loaded with 186 L of cold tap city water and 80 kg corn starch type Cerestar C x PHARM 03406. The feed mixer was kept stirred gentle and pH was adjusted to 4.5 using 30 % HCl. The temperature was kept at 7-8°C using cooling water and the enzymes CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch) was added. The low temperature secured that no reaction took place.

The upstart of the reactor was continued until the °Brix-value after 30 hours had stabilized around 27. Then the microfiltration was initiated using a pressure drop of 0.15 Bar and maximal retentate flow to secure this pressure. The filtrate was recycled to the reactor tank the first 5.7 hours. Hereafter the filtrate was collected in a separate tank, and the volume was measured as a function of time. At this point of time the reactor feed pump was started and adjusted to a flow rate equivalent to the filtrate flux (L/min). By doing so the volume in the reactor tank was kept constant.

The feed of starch slurry was continued while samples were taken as described above. Furthermore samples of the filtrates were taken. Any decrease in the filtrate flux were compensated for by increasing the retentate flow whereby the filter cake on the membrane was disrupted. Thereby the pressure drop was increased too. Samples were taken as a function of time of the filtrate for HPLC and °BRIX as well as the volume collected was measured. Simultaneously samples were taken from the reactor for measuring of total DS, sludge, °Brix and HPLC for sugar composition.

The trial lasted 220 hours. At that point of time the pressure drop was increased to about 0.4 Bar.

Determination of filtrate flux (based on single determinations) and average filtrate flux values (integrated) as a function of the process time showed that the enzyme system consisting of a CGTase and a glucoamylase alone maintained and secured a stable flux over a long processing time. This underlines the industrial potential advantages of this stable system.

The results and a mass balance are presented in tables 16-18.

Table 16. Analyses of collected filtrates.

Date and time	Hours from start	Collected filtrate, L	% DS w/w	Density, kg/L	Mass of DS, kg	Average flux, mL/min
13/03/02 16:05	30*	-	-	-	-	-
14/03/02 16:50	55	142	25.8	1.12	41.1	95.6
16/03/02 16:00	102	187	25.6	1.12	53.7	66.1
18/03/02 13:02	147	200	28.7	1.14	65.2	74.0
19/03/02 16:45	174	100	29.6	1.14	33.8	60.1
Total collected		629.0	27.3	1.13	193.7	-

*Start of continuous feeding to the reactor

Table 17. Composition of the syrup produced

% DP1	%DP2	% DP3	%DP4
93	5	1	2

Table 18. Mass balance for the trial of example 7

	Mass, kg	% DS	Mass of DS, kg	% Yield of DS*
Upstart of reactor				
Starch	60	90	54	25
Water	140	0	0	
Reactor start	200	27.0	54	25
Continuous production				
Starch consumption (t=28.75 h to t=174.5 h)	235.48	90	212	100
Water consumption (t=28.75 h to t=174.5 h)	548.12	0	0	
Substrate consumption	783.6	27.0	212	100
Syrup production	629.0	27.3	172	81
Reactor at end				
Total content	200	35	70	33
Unconverted starch	18	50	9	4
Mud, L	18	50	9	4
Glucose syrup	164	30	49	23

*basis substrate consumption at continuous production.

Compared to a batch trial carried out in a simple tank with stirring a significant reduction of the reaction time was obtained using the setup for hydrolysis of granular starch described above. As no viscosity problems were encountered with 30% DS it is considered feasible to increase the DS to 40%, or even as high as 45% and still maintain a smooth operation.

Example 8

This example compares a process of the invention and a conventional process for production of fuel ethanol or potable alcohol from raw starch in the form of dry milled corn, Yellow Dent No. 2.

A slurry of 30 % DS of dry milled corn was prepared in tap water in 250 ml blue cap flasks and the raw corn starch exposed to simultaneous cold liquefaction and pre-saccharification by a process of the invention. The slurry was heated to 60 °C in a water bath under magnet stirring, pH adjusted to 4.5 using 30 % HCl and CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS) added. After 48 hours the flask was cooled in the water bath to 32 °C.

A slurry of 30 % DS dry milled corn was pre-liquefied in a conventional continuous process consisting of a pre-liquefaction vessel, a jet-cooker, a flash, and a post liquefaction vessel. *Bacillus* alpha-amylase was added during the pre-liquefaction at 70-90°C (10 KNU/kg DS) and again during the post liquefaction at ca. 85-90°C (20 KNU/kg DS). The jet-cooking was carried out at 115-120°C. Pre-saccharification was performed under magnet stirring by

heating the mash in blue cap flasks to 60 °C in a water bath. After pH adjustment to 4.5 using 30 % HCl glucoamylase was added in a dosage equivalent to 500 AGU/kg DS. After 48 hours the flask was cooled in the water bath to 32 °C.

Fermentations were made directly in the blue cap flasks fitted with yeast locks filled with soybean oil. Bakers yeast (*Saccharomyces cerevisiae*) was added in an amount equivalent to 10 millions/mL of viable yeast cells and yeast nutrition in the form of 0.25 % urea was added to each flask. Each treatment was performed in 3 replicates.

The fermentation was monitored by the CO_2 loss as determined by weighing the flasks at regular intervals. L EtOH/100 kg grain dry matter (DS) was then calculated using the following formula:

$$\text{L EtOH/100 kg mash dry matter} = \frac{\text{Weight loss (g)} \times 1.045}{0.79\,\text{(g/mL)} \times 250 \times 30\%\ \text{dry matter}} \times 100$$

The mash contained 30 % w/w grain dry matter. 0.79 g/mL is the density of ethanol.

Tables 19 and 20 shows the obtained fermentation results for the replicates including the results of statistical calculation of the two types of pretreated raw materials (missing results estimated by interpolation).

Table 19. Fermentation result for the process of the invention using CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS).

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	28,3	0.9
48	35,4	0.6
69	37,1	0.2
79	*37,5	-
97	38,3	0.2

*Estimated value

Table 20. Fermentation result for a conventional process using *Bacillus* alpha-amylase (10+20 KNU/kg DS) and glucoamylase (500 AGU/kg DS)

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	22,5	1.3
48	33,9	0.7
69	*37,2	-
79	38,8	0.4
97	40,5	0.5

*Estimated value

Using a simulated industrial fermentation time in the interval of approximately 48-70 hours an equivalent or higher alcohol yield was obtained from the mash produced by the process of the invention than could be obtained from a mash produced by the more energy consuming two step hot slurry pre-liquefying and jet-cooking process.

Example 9

This example illustrates the conversion of granular wheat and common corn starch into glucose using a CGTase, a glucoamylase and an acid fungal alpha-amylase at 60°C.

Flasks with either 33% DS common corn or wheat granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 20 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 21 and table 22.

Table 20. The enzyme activity levels used were:

CGTase	Glucoamylase	Acid fungal alpha-amylase
NU/g DS	AGU/g DS	AFAU/g DS
100.0	0.2	0.05

Table 21. Soluble dry solids as percentage of total dry substance using two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	85.9	96.2	99.4	100.0
Wheat	95.7	98.9	99.6	100.0

Table 22. The DX of the soluble hydrolysate using the two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	76.2	89.2	93.4	94.7
Wheat	86.2	92.4	93.6	94.4

CLAIMS

1. A one step process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the simultaneous action of;

   a first enzyme which;

   (a) is a member of the Glycoside Hydrolase Family13;

   (b) has alpha-1.4-glucosidic hydrolysis activity, and;

   (c) comprises a Carbohydrate-Binding Module Family 20,

   and at least one second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), or a glucoamylase (E.C.3.2.1.3).

2. The process of the preceding claim, wherein the starch slurry has 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids, especially around 33% dry solids granular starch.

3. The process of any of the preceding claims, wherein at least 85%, 86% , 87%, 88%, 89% least 90%, 91%, 92%, 93% 94%, 95%, 96%, 97%, 98% or at least 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

4. The process of any of the preceding claims, wherein the first enzyme is of microbial origin, and preferably of bacterial origin.

5. The process of any of the preceding claims, wherein the first enzyme is a CGTase (EC 2.4.1.19).

6. The process of any of the preceding claims, wherein the first enzyme is a CGTase having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per min/mg.

7. The process of any of the preceding claims, wherein the first enzyme is a CGTase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in figure 1 in Joergensen et al. (1997), Biotechnol. Lett. 19:1027-1031.

8. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase (E.C. 3.2.1.133).

9. The process of any of the preceding claims, wherein the maltogenic alpha-amylase is derived from *Bacillus*, preferably from *B. stearothermophilus*.

10. The process of any of the preceding claims wherein the first enzyme is a maltogenic alpha-amylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:1 in WO9943794.

11. The process of any of the preceding claims, wherein the first enzyme is the maltogenic alpha-amylase having the amino acid sequence shown SEQ ID NO:1 in WO9943794 or a variant of said amino acid sequence disclosed in said patent.

12. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.

13. The process of any of the preceding claims, wherein the second enzyme is a fungal alpha-amylase, having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:10 in WO9623874.

14. The process of any of the preceding claims, wherein the second enzyme is a barley beta-amylase (E.C. 2.4.1.2), such as Spezyme® BBA 1500 or Spezyme® DBA from Genencor int.

15. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase.

16. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136.

17. The process of any of the preceding claims wherein a third enzyme is present, said third enzyme being an alpha-amylase derived from a *Bacillus* sp., such as the enzymes, the variants and hybrids disclosed in WO99/19467, WO96/23874, WO97/41213, and WO99/19467.

18. The process of any of the preceding claims, wherein a third enzyme is present, said enzyme being an isoamylase or a pullulanase.

19. The process of any of the preceding claims, wherein the temperature is at least 58°C, 59°C, or more preferably at least 60°C.

20. The process of any of the preceding claims, wherein the pH is in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

21. The process of any of the preceding claims, wherein the soluble starch hydrolysate has a DX of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or at least 99.5%.

22. The process of any of the preceding claims, wherein the dominating saccharide in the soluble starch hydrolysate is glucose or maltose.

23. The process of any of the preceding claims, wherein the granular starch is obtained from tubers, roots, stems, or whole grain.

24. The process of any of the preceding claims, wherein the granular starch is obtained from cereals.

25. The process of any of the preceding claims, wherein the granular starch is obtained from corn, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice or potatoes.

26. The process of any of the preceding claims, wherein the granular starch is obtained from dry milling of whole grain or from wet milling of whole grain.

27. The process of any of the preceding claims, wherein the process is conducted in an ultrafiltration system and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

28. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

29. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

30. A process for production of high fructose starch-based syrup (HFSS), wherein a soluble starch hydrolysate of the process of any of the preceding claims is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS).

31. A process for production of fuel or potable ethanol, wherein a soluble starch hydrolysate of the process of any of claims 1-29 is subjected to fermentation into ethanol.

32. The process of claim 31, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.

33. The process of any of the claims 31-32, wherein the process is conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

34. The process of any of the claims 31-33, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

10270-000-WO SEQ LIST.ST25.txt
SEQUENCE LISTING

<110> Novozymes

<120> Cold liquefaction

<130> 10270-WO

<160> 4

<170> PatentIn version 3.2

<210> 1
<211> 706
<212> PRT
<213> Bacillus

<220>
<221> mat_peptide
<222> (29)..()

<400> 1

```
Val Phe Leu Lys Asn Leu Thr Val Leu Leu Lys Thr Ile Pro Leu Ala
           -25                 -20                 -15

Leu Leu Leu Phe Ile Leu Leu Ser Leu Pro Thr Ala Ala Gln Ala Asp
        -10                 -5                  -1  1

Val Thr Asn Lys Val Asn Tyr Thr Arg Asp Val Ile Tyr Gln Ile Val
5                   10                  15                  20

Thr Asp Arg Phe Ser Asp Gly Asp Pro Ser Asn Asn Pro Thr Gly Ala
                25                  30                  35

Ile Tyr Ser Gln Asp Cys Ser Asp Leu His Lys Tyr Cys Gly Gly Asp
            40                  45                  50

Trp Gln Gly Ile Ile Asp Lys Ile Asn Asp Gly Tyr Leu Thr Asp Leu
        55                  60                  65

Gly Ile Thr Ala Ile Trp Ile Ser Gln Pro Val Glu Asn Val Tyr Ala
    70                  75                  80

Leu His Pro Ser Gly Tyr Thr Ser Tyr His Gly Tyr Trp Ala Arg Asp
85                  90                  95                  100

Tyr Lys Arg Thr Asn Pro Phe Tyr Gly Asp Phe Ser Asp Phe Asp Arg
                105                 110                 115

Leu Met Asp Thr Ala His Ser Asn Gly Ile Lys Val Ile Met Asp Phe
            120                 125                 130

Thr Pro Asn His Ser Ser Pro Ala Leu Glu Thr Asp Pro Ser Tyr Ala
        135                 140                 145
```

Glu Asn Gly Ala Val Tyr Asn Asp Gly Val Leu Ile Gly Asn Tyr Ser
150 155 160

Asn Asp Pro Asn Asn Leu Phe His His Asn Gly Gly Thr Asp Phe Ser
165 170 175 180

Ser Tyr Glu Asp Ser Ile Tyr Arg Asn Leu Tyr Asp Leu Ala Asp Tyr
185 190 195

Asp Leu Asn Asn Thr Val Met Asp Gln Tyr Leu Lys Glu Ser Ile Lys
200 205 210

Leu Trp Leu Asp Lys Gly Ile Asp Gly Ile Arg Val Asp Ala Val Lys
215 220 225

His Met Ser Glu Gly Trp Gln Thr Ser Leu Met Ser Asp Ile Tyr Ala
230 235 240

His Glu Pro Val Phe Thr Phe Gly Glu Trp Phe Leu Gly Ser Gly Glu
245 250 255 260

Val Asp Pro Gln Asn His His Phe Ala Asn Glu Ser Gly Met Ser Leu
265 270 275

Leu Asp Phe Gln Phe Gly Gln Thr Ile Arg Asp Val Leu Met Asp Gly
280 285 290

Ser Ser Asn Trp Tyr Asp Phe Asn Glu Met Ile Ala Ser Thr Glu Glu
295 300 305

Asp Tyr Asp Glu Val Ile Asp Gln Val Thr Phe Ile Asp Asn His Asp
310 315 320

Met Ser Arg Phe Ser Phe Glu Gln Ser Ser Asn Arg His Thr Asp Ile
325 330 335 340

Ala Leu Ala Val Leu Leu Thr Ser Arg Gly Val Pro Thr Ile Tyr Tyr
345 350 355

Gly Thr Glu Gln Tyr Leu Thr Gly Gly Asn Asp Pro Glu Asn Arg Lys
360 365 370

Pro Met Ser Asp Phe Asp Arg Thr Thr Asn Ser Tyr Gln Ile Ile Ser
375 380 385

Thr Leu Ala Ser Leu Arg Gln Ser Asn Pro Ala Leu Gly Tyr Gly Asn
390 395 400

Thr Ser Glu Arg Trp Ile Asn Ser Asp Val Tyr Ile Tyr Glu Arg Ala
405 410 415 420

Phe Gly Asp Ser Val Val Leu Thr Ala Val Asn Ser Gly Asp Thr Ser
425 430 435

Tyr Thr Ile Asn Asn Leu Asn Thr Ser Leu Pro Gln Gly Gln Tyr Thr
440 445 450

Asp Glu Leu Gln Gln Leu Leu Asp Gly Asn Glu Ile Thr Val Asn Ser
455 460 465

Asn Gly Ala Val Asp Ser Phe Gln Leu Ser Ala Asn Gly Val Ser Val
470 475 480

Trp Gln Ile Thr Glu Glu His Ala Ser Pro Leu Ile Gly His Val Gly
485 490 495 500

Pro Met Met Gly Lys His Gly Asn Thr Val Thr Ile Thr Gly Glu Gly
505 510 515

Phe Gly Asp Asn Glu Gly Ser Val Leu Phe Asp Ser Asp Phe Ser Asp
520 525 530

Val Leu Ser Trp Ser Asp Thr Lys Ile Glu Val Ser Val Pro Asp Val
535 540 545

Thr Ala Gly His Tyr Asp Ile Ser Val Val Asn Ala Gly Asp Ser Gln
550 555 560

Ser Pro Thr Tyr Asp Lys Phe Glu Val Leu Thr Gly Asp Gln Val Ser
565 570 575 580

Ile Arg Phe Ala Val Asn Asn Ala Thr Thr Ser Leu Gly Thr Asn Leu
585 590 595

Tyr Met Val Gly Asn Val Asn Glu Leu Gly Asn Trp Asp Pro Asp Gln
600 605 610

Ala Ile Gly Pro Met Phe Asn Gln Val Met Tyr Gln Tyr Pro Thr Trp
615 620 625

Tyr Tyr Asp Ile Ser Val Pro Ala Glu Glu Asn Leu Glu Tyr Lys Phe
630 635 640

Ile Lys Lys Asp Ser Ser Gly Asn Val Val Trp Glu Ser Gly Asn Asn
645 650 655 660

His Thr Tyr Thr Thr Pro Ala Thr Gly Thr Asp Thr Val Leu Val Asp
665 670 675

Trp Gln

<210> 2
<211> 705
<212> PRT
<213> Bacillus

<220>
<221> mat_peptide
<222> (32)..()

<400> 2

```
Met Leu Asn Lys Leu Ser Leu Lys Met Lys Ala Ile Ala Phe Phe Gly
    -30                 -25                 -20

Ile Val Phe Val Val Phe Leu Ala Leu Ala Asn Asp Val Tyr Ala Ala
-15                 -10                 -5                  -1  1

Asn Gln Leu Asn Lys Val Asn Tyr Ala Lys Asp Thr Ile Tyr Gln Ile
            5                   10                  15

Val Thr Asp Arg Phe Leu Asp Gly Asp Pro Ser Asn Asn Pro Asp Gly
        20                  25                  30

Ala Leu Tyr Ser Glu Thr Asp Leu His Lys Tyr Met Gly Gly Asp Trp
    35                  40                  45

Lys Gly Ile Thr Glu Lys Ile Glu Asp His Tyr Phe Thr Asp Leu Gly
50                  55                  60                  65

Ile Thr Ala Leu Trp Ile Ser Gln Pro Val Glu Asn Val Tyr Ala Val
                70                  75                  80

His Pro Glu Gly Tyr Thr Ser Tyr His Gly Tyr Trp Ala Arg Asp Tyr
            85                  90                  95

Lys Lys Thr Asn Pro Phe Tyr Gly Asn Phe Asn Asp Phe Asp Glu Leu
        100                 105                 110

Ile Ser Thr Ala His Ser His Gly Ile Lys Ile Ile Met Asp Phe Thr
    115                 120                 125

Pro Asn His Ser Ser Pro Ala Leu Lys Thr Asp Ser Asp Tyr Val Glu
130                 135                 140                 145

Asn Gly Ala Ile Tyr Asp Asn Gly Ser Leu Ile Gly Asn Tyr Ser Asn
                150                 155                 160

Asp Leu Asp Ile Phe His His Asn Gly Gly Thr Asp Phe Ser Ser Tyr
            165                 170                 175

Glu Asp Gly Ile Tyr Arg Asn Leu Tyr Asp Leu Ala Asp Tyr Asp Leu
        180                 185                 190
```

```
Gln Asn Gln Thr Ile Asp Gln Tyr Leu Lys Glu Ser Ile Glu Leu Trp
    195                 200                 205

Leu Asp Lys Gly Ile Asp Gly Ile Arg Val Asp Ala Val Lys His Met
210                 215                 220                 225

Ser Gln Gly Trp Gln Glu Thr Leu Thr Asn His Ile Tyr Ser Tyr Gln
                230                 235                 240

Pro Val Phe Thr Phe Gly Glu Trp Phe Leu Gly Glu Asn Glu Ile Asp
            245                 250                 255

Pro Arg Asn His Tyr Phe Ala Asn Glu Ser Gly Met Ser Leu Leu Asp
        260                 265                 270

Phe Gln Phe Gly Gln Gln Ile Arg Gly Val Leu Met Ser Gln Glu Asp
    275                 280                 285

Asp Trp Thr Asp Phe His Thr Met Ile Glu Asp Thr Ser Asn Ser Tyr
290                 295                 300                 305

Asn Glu Val Ile Asp Gln Val Thr Phe Ile Asp Asn His Asp Met Ser
                310                 315                 320

Arg Phe His Lys Glu Asp Gly Ala Lys Thr Asn Thr Asp Ile Ala Leu
            325                 330                 335

Ala Val Leu Leu Thr Ser Arg Gly Val Pro Thr Ile Tyr Tyr Gly Thr
        340                 345                 350

Glu His Tyr Leu Thr Gly Glu Ser Asp Pro Glu Asn Arg Lys Pro Met
    355                 360                 365

Pro Ser Phe Asp Arg Ala Thr Thr Ala Tyr Gln Ile Ile Ser Lys Leu
370                 375                 380                 385

Ala His Leu Arg Gln Ser Asn Pro Ala Leu Gly Tyr Gly Thr Thr Thr
                390                 395                 400

Glu Arg Trp Leu Asn Glu Asp Val Tyr Ile Phe Glu Arg Lys Phe Gly
            405                 410                 415

Asp Asn Val Val Val Thr Ala Val Asn Ser Gly Glu Gln Ser Tyr Thr
        420                 425                 430

Ile Asn Asn Leu Gln Thr Ser Leu Leu Glu Gly Thr His Pro Asp Val
    435                 440                 445

Leu Glu Gly Leu Met Gly Gly Asp Ala Leu Gln Ile Asp Gly Lys Gly
450                 455                 460                 465
```

Gln Ala Ser Thr Phe Glu Leu Lys Ala Asn Glu Val Ala Val Trp Glu
470 475 480

Val Thr Ala Glu Ser Asn Thr Pro Leu Ile Gly His Val Gly Pro Met
485 490 495

Val Gly Gln Ala Gly Asn Glu Ile Thr Ile Ser Gly Glu Gly Phe Gly
500 505 510

Glu Gly Gln Gly Thr Val Leu Phe Gly Ser Asp Gln Ala Ser Ile Val
515 520 525

Ser Trp Gly Asp Ser Glu Ile Val Val Asn Val Pro Asp Arg Pro Gly
530 535 540 545

Asn His Tyr Asn Ile Glu Val Val Thr Asn Asp Asn Lys Glu Ser Asn
550 555 560

Pro Tyr Ser Asp Phe Glu Ile Leu Thr Asn Lys Leu Ile Pro Val Arg
565 570 575

Phe Ile Val Glu Glu Ala Val Thr Asp Tyr Gly Thr Ser Val Tyr Leu
580 585 590

Val Gly Asn Thr Gln Glu Leu Gly Asn Trp Asp Thr Asp Lys Ala Ile
595 600 605

Gly Pro Phe Phe Asn Gln Ile Ile Ala Gln Tyr Pro Thr Trp Tyr Tyr
610 615 620 625

Asp Ile Ser Val Pro Ala Asp Ser Thr Leu Glu Tyr Lys Phe Ile Lys
630 635 640

Lys Asp Ala Leu Gly Asn Val Val Trp Glu Ser Gly Thr Asn Arg Ser
645 650 655

Tyr Glu Thr Pro Thr Glu Gly Thr Asp Thr Leu Thr Ser Thr Trp Arg
660 665 670

Asn

<210> 3
<211> 683
<212> PRT
<213> Thermoanaerobacter sp.

<220>
<221> mat_peptide
<222> (1)..()

<400> 3

```
Ala Pro Asp Thr Ser Val Ser Asn Val Val Asn Tyr Ser Thr Asp Val
1               5                  10                  15

Ile Tyr Gln Ile Val Thr Asp Arg Phe Leu Asp Gly Asn Pro Ser Asn
            20                  25                  30

Asn Pro Thr Gly Asp Leu Tyr Asp Pro Thr His Thr Ser Leu Lys Lys
        35                  40                  45

Tyr Phe Gly Gly Asp Trp Gln Gly Ile Ile Asn Lys Ile Asn Asp Gly
    50                  55                  60

Tyr Leu Thr Gly Met Gly Ile Thr Ala Ile Trp Ile Ser Gln Pro Val
65                  70                  75                  80

Glu Asn Ile Tyr Ala Val Leu Pro Asp Ser Thr Phe Gly Gly Ser Thr
                85                  90                  95

Ser Tyr His Gly Tyr Trp Ala Arg Asp Phe Lys Lys Thr Asn Pro Phe
            100                 105                 110

Phe Gly Ser Phe Thr Asp Phe Gln Asn Leu Ile Ala Thr Ala His Ala
        115                 120                 125

His Asn Ile Lys Val Ile Ile Asp Phe Ala Pro Asn His Thr Ser Pro
    130                 135                 140

Ala Ser Glu Thr Asp Pro Thr Tyr Gly Glu Asn Gly Arg Leu Tyr Asp
145                 150                 155                 160

Asn Gly Val Leu Leu Gly Gly Tyr Thr Asn Asp Thr Asn Gly Tyr Phe
                165                 170                 175

His His Tyr Gly Gly Thr Asn Phe Ser Ser Tyr Glu Asp Gly Ile Tyr
            180                 185                 190

Arg Asn Leu Phe Asp Leu Ala Asp Leu Asp Gln Gln Asn Ser Thr Ile
        195                 200                 205

Asp Ser Tyr Leu Lys Ala Ala Ile Lys Leu Trp Leu Asp Met Gly Ile
    210                 215                 220

Asp Gly Ile Arg Met Asp Ala Val Lys His Met Ala Phe Gly Trp Gln
225                 230                 235                 240

Lys Asn Phe Met Asp Ser Ile Leu Ser Tyr Arg Pro Val Phe Thr Phe
                245                 250                 255

Gly Glu Trp Tyr Leu Gly Thr Asn Glu Val Asp Pro Asn Asn Thr Tyr
            260                 265                 270
```

Phe Ala Asn Glu Ser Gly Met Ser Leu Leu Asp Phe Arg Phe Ala Gln
275 280 285

Lys Val Arg Gln Val Phe Arg Asp Asn Thr Asp Thr Met Tyr Gly Leu
290 295 300

Asp Ser Met Ile Gln Ser Thr Ala Ala Asp Tyr Asn Phe Ile Asn Asp
305 310 315 320

Met Val Thr Phe Ile Asp Asn His Asp Met Asp Arg Phe Tyr Thr Gly
325 330 335

Gly Ser Thr Arg Pro Val Glu Gln Ala Leu Ala Phe Thr Leu Thr Ser
340 345 350

Arg Gly Val Pro Ala Ile Tyr Tyr Gly Thr Glu Gln Tyr Met Thr Gly
355 360 365

Asn Gly Asp Pro Tyr Asn Arg Ala Met Met Thr Ser Phe Asp Thr Thr
370 375 380

Thr Thr Ala Tyr Asn Val Ile Lys Lys Leu Ala Pro Leu Arg Lys Ser
385 390 395 400

Asn Pro Ala Ile Ala Tyr Gly Thr Gln Lys Gln Arg Trp Ile Asn Asn
405 410 415

Asp Val Tyr Ile Tyr Glu Arg Gln Phe Gly Asn Asn Val Ala Leu Val
420 425 430

Ala Ile Asn Arg Asn Leu Ser Thr Ser Tyr Tyr Ile Thr Gly Leu Tyr
435 440 445

Thr Ala Leu Pro Ala Gly Thr Tyr Ser Asp Met Leu Gly Gly Leu Leu
450 455 460

Asn Gly Ser Ser Ile Thr Val Ser Ser Asn Gly Ser Val Thr Pro Phe
465 470 475 480

Thr Leu Ala Pro Gly Glu Val Ala Val Trp Gln Tyr Val Ser Thr Thr
485 490 495

Asn Pro Pro Leu Ile Gly His Val Gly Pro Thr Met Thr Lys Ala Gly
500 505 510

Gln Thr Ile Thr Ile Asp Gly Arg Gly Phe Gly Thr Thr Ala Gly Gln
515 520 525

Val Leu Phe Gly Thr Thr Pro Ala Thr Ile Val Ser Trp Glu Asp Thr
530 535 540

```
Glu Val Lys Val Lys Val Pro Ala Leu Thr Pro Gly Lys Tyr Asn Ile
545                 550                 555                 560

Thr Leu Lys Thr Ala Ser Gly Val Thr Ser Asn Ser Tyr Asn Asn Ile
                565                 570                 575

Asn Val Leu Thr Gly Asn Gln Val Cys Val Arg Phe Val Val Asn Asn
            580                 585                 590

Ala Thr Thr Val Trp Gly Glu Asn Val Tyr Leu Thr Gly Asn Val Ala
        595                 600                 605

Glu Leu Gly Asn Trp Asp Thr Ser Lys Ala Ile Gly Pro Met Phe Asn
    610                 615                 620

Gln Val Val Tyr Gln Tyr Pro Thr Trp Tyr Tyr Asp Val Ser Val Pro
625                 630                 635                 640

Ala Gly Thr Thr Ile Glu Phe Lys Phe Ile Lys Lys Asn Gly Ser Thr
                645                 650                 655

Val Thr Trp Glu Gly Gly Tyr Asn His Val Tyr Thr Thr Pro Thr Ser
            660                 665                 670

Gly Thr Ala Thr Val Ile Val Asp Trp Gln Pro
        675                 680
```

<210> 4
<211> 713
<212> PRT
<213> Bacillus

<220>
<221> mat_peptide
<222> (28)..()

<400> 4

```
Met Lys Arg Phe Met Lys Leu Thr Ala Val Trp Thr Leu Trp Leu Ser
        -25                 -20                 -15

Leu Thr Leu Gly Leu Leu Ser Pro Val His Ala Ala Pro Asp Thr Ser
    -10                 -5                  -1  1               5

Val Ser Asn Lys Gln Asn Phe Ser Thr Asp Val Ile Tyr Gln Ile Phe
                10                  15                  20

Thr Asp Arg Phe Ser Asp Gly Asn Pro Ala Asn Asn Pro Thr Gly Ala
            25                  30                  35

Ala Phe Asp Gly Ser Cys Thr Asn Leu Arg Leu Tyr Cys Gly Gly Asp
        40                  45                  50
```

Trp Gln Gly Ile Ile Asn Lys Ile Asn Asp Gly Tyr Leu Thr Gly Met
55 60 65

Gly Ile Thr Ala Ile Trp Ile Ser Gln Pro Val Glu Asn Ile Tyr Ser
70 75 80 85

Val Ile Asn Tyr Ser Gly Val Asn Asn Thr Ala Tyr His Gly Tyr Trp
90 95 100

Ala Arg Asp Phe Lys Lys Thr Asn Pro Ala Tyr Gly Thr Met Gln Asp
105 110 115

Phe Lys Asn Leu Ile Asp Thr Ala His Ala His Asn Ile Lys Val Ile
120 125 130

Ile Asp Phe Ala Pro Asn His Thr Ser Pro Ala Ser Ser Asp Asp Pro
135 140 145

Ser Phe Ala Glu Asn Gly Arg Leu Tyr Asp Asn Gly Asn Leu Leu Gly
150 155 160 165

Gly Tyr Thr Asn Asp Thr Gln Asn Leu Phe His His Tyr Gly Gly Thr
170 175 180

Asp Phe Ser Thr Ile Glu Asn Gly Ile Tyr Lys Asn Leu Tyr Asp Leu
185 190 195

Ala Asp Leu Asn His Asn Asn Ser Ser Val Asp Val Tyr Leu Lys Asp
200 205 210

Ala Ile Lys Met Trp Leu Asp Leu Gly Val Asp Gly Ile Arg Val Asp
215 220 225

Ala Val Lys His Met Pro Phe Gly Trp Gln Lys Ser Phe Met Ala Thr
230 235 240 245

Ile Asn Asn Tyr Lys Pro Val Phe Thr Phe Gly Glu Trp Phe Leu Gly
250 255 260

Val Asn Glu Ile Ser Pro Glu Tyr His Gln Phe Ala Asn Glu Ser Gly
265 270 275

Met Ser Leu Leu Asp Phe Arg Phe Ala Gln Lys Ala Arg Gln Val Phe
280 285 290

Arg Asp Asn Thr Asp Asn Met Tyr Gly Leu Lys Ala Met Leu Glu Gly
295 300 305

Ser Glu Val Asp Tyr Ala Gln Val Asn Asp Gln Val Thr Phe Ile Asp
310 315 320 325

Asn His Asp Met Glu Arg Phe His Thr Ser Asn Gly Asp Arg Arg Lys
330 335 340

Leu Glu Gln Ala Leu Ala Phe Thr Leu Thr Ser Arg Gly Val Pro Ala
345 350 355

Ile Tyr Tyr Gly Ser Glu Gln Tyr Met Ser Gly Gly Asn Asp Pro Asp
360 365 370

Asn Arg Ala Arg Leu Pro Ser Phe Ser Thr Thr Thr Thr Ala Tyr Gln
375 380 385

Val Ile Gln Lys Leu Ala Pro Leu Arg Lys Ser Asn Pro Ala Ile Ala
390 395 400 405

Tyr Gly Ser Thr His Glu Arg Trp Ile Asn Asn Asp Val Ile Ile Tyr
410 415 420

Glu Arg Lys Phe Gly Asn Asn Val Ala Val Val Ala Ile Asn Arg Asn
425 430 435

Met Asn Thr Pro Ala Ser Ile Thr Gly Leu Val Thr Ser Leu Arg Arg
440 445 450

Ala Ser Tyr Asn Asp Val Leu Gly Gly Ile Leu Asn Gly Asn Thr Leu
455 460 465

Thr Val Gly Ala Gly Gly Ala Ala Ser Asn Phe Thr Leu Ala Pro Gly
470 475 480 485

Gly Thr Ala Val Trp Gln Tyr Thr Thr Asp Ala Thr Thr Pro Ile Ile
490 495 500

Gly Asn Val Gly Pro Met Met Ala Lys Pro Gly Val Thr Ile Thr Ile
505 510 515

Asp Gly Arg Gly Phe Gly Ser Gly Lys Gly Thr Val Tyr Phe Gly Thr
520 525 530

Thr Ala Val Thr Gly Ala Asp Ile Val Ala Trp Glu Asp Thr Gln Ile
535 540 545

Gln Val Lys Ile Pro Ala Val Pro Gly Gly Ile Tyr Asp Ile Arg Val
550 555 560 565

Ala Asn Ala Ala Gly Ala Ala Ser Asn Ile Tyr Asp Asn Phe Glu Val
570 575 580

Leu Thr Gly Asp Gln Val Thr Val Arg Phe Val Ile Asn Asn Ala Thr
585 590 595

Thr Ala Leu Gly Gln Asn Val Phe Leu Thr Gly Asn Val Ser Glu Leu
600 605 610

Gly Asn Trp Asp Pro Asn Asn Ala Ile Gly Pro Met Tyr Asn Gln Val
615 620 625

Val Tyr Gln Tyr Pro Thr Trp Tyr Tyr Asp Val Ser Val Pro Ala Gly
630 635 640 645

Gln Thr Ile Glu Phe Lys Phe Leu Lys Lys Gln Gly Ser Thr Val Thr
650 655 660

Trp Glu Gly Gly Ala Asn Arg Thr Phe Thr Thr Pro Thr Ser Gly Thr
665 670 675

Ala Thr Val Asn Val Asn Trp Gln Pro
680 685